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Distribution of connexin37, connexin40 and connexin43 in the aorta and coronary artery of several mammals

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Abstract Intercellular communication between cells of the vessel wall is established by a combination of diffusion and convection of humoral and endothelial factors in the extracellular fluid or by direct intercellular contacts present in the form of gap junctions composed of proteins called connexins. At least connexin (Cx)37, Cx40 and Cx43 are expressed in the vessel wall, but disparate findings with regard to the cell specific localisation of connexins in the vasculature indicate that the distribution of connexins may be species and vessel specific. Moreover, differences in expression exist between cells in culture and tissue sections. We performed an inventory immunohistochemical study on the localisation of Cx37, Cx40 and Cx43 on tissue sections of the bovine, micropig and rat aorta and coronary system, which represent morphologically and functionally different types of vessels in the arterial system. We could observe Cx40 labelling most commonly, although with various intensities, between endothelial and smooth muscle cells of the species studied, with the exception of rat aortic smooth muscle cells. The distribution of Cx43 is more differentiated and mostly confined to smooth muscle cells, although it can be detected scarcely between endothelial cells. Cx37, when detectable, is predominantly expressed between endothelial cells in a heterogeneous pattern. We conclude that Cx40 is the constitutive vascular gap junction protein in situ and guarantees cell coupling between cells in the vessel wall. The differentiated distribution of both Cx37 and Cx43 suggests they are involved in more dynamic processes.

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Introduction

In healthy vasculature, tissue homeostasis and vasomotor tone greatly depends on the intercellular communication between endothelial cells and smooth muscle cells. This is established either by diffusion and convection of humoral and endothelial factors in the extracellular fluid or by direct intercellular contacts present in the form of gap junctions.

Gap junctions are clusters of transmembrane channels joining the cytoplasm of neighbouring cells (Gros and Jongsma 1996). They allow passage of ions, second messengers and metabolites up to about 1 kDa. Gap junctions are commonly present between virtually all mammalian cell types. One gap junction channel in a plaque is formed by head to head alignment of two half channels, named connexons, contributed by each of the two participating cells. Each connexon is a hexameric assembly of transmembrane proteins called connexins. Connexins comprise a multigene family of proteins consisting of at least 14 members, named according to their molecular weight predicted from the cDNA sequences. Within one organ or cell type various different connexins can be expressed. It is not yet well established what the functional implications of this connexin diversity can be. Furthermore it is not known to what extent different connexins are combined within connexons and channels. It is known that in mammalian vasculature at least connexin (Cx)37, Cx40 and Cx43 are expressed (Bruzzone et al. 1993; Haefliger et al. 1992; Hennemann et al. 1992; Larson and Haudenschild 1990; Little et al. 1995; Reed et al. 1993). However, apparent controversial findings with regard to the cell-specific localisation of connexins in the vessel wall indicate that the distribution of connexins may be species and vessel specific. We performed an inventory immunohistochemical study on the localisation of Cx37, Cx40 and Cx43 in parts of the bovine, micropig and rat aorta and coronary system. They represent morphologically and functionally different types of vessels in the arterial system. The aorta exemplifies the category of conduit vessels, which contain large amounts of

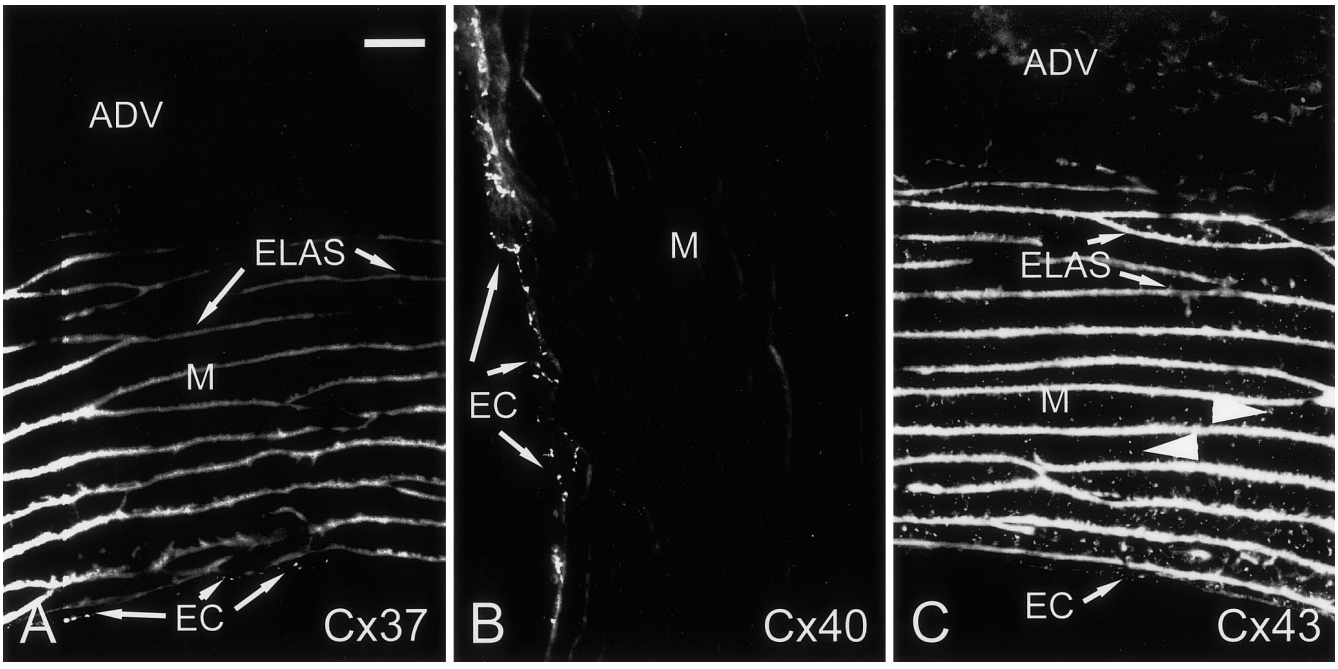

Fig. 1A–C Serial sections of rat aorta. Gap junction labelling is represented by *white dots* and *speckles*. Elastic fibres (*ELAS*) can be distinguished as autofluorescent lines. The photomicrographs show the intima with endothelial cells (*EC*), the media (*M*) and the adventitia (*ADV*). Between the endothelial cells, all three connexin isotypes can be distinguished (**A–C** *arrows*). Between the smooth muscle cells within the media, only Cx43 can be detected (**C**). *Bar* 25 µm

elastic tissue to accommodate passive pulsatile blood flow. The coronary system exemplifies the category of resistance vessels with a relative greater prominence of the smooth muscle component and a more active role in blood flow. Choice of species studied is determined by size of the animal (rat versus cow) and usage as an animal model for atherosclerosis (rat versus micropig).

Materials and methods

Tissue samples and processing

Bovine hearts (*n*=3) were freshly isolated in the regional slaughterhouse and pieces of the proximal aorta and the coronary system (right coronary artery; RCA, left coronary artery; LCA, circumflex artery) were dissected within 4 h, embedded in Tissue Tek and directly frozen in liquid nitrogen. Yucatan micropigs (*n*=3) where anaesthetised for the purpose of angiography necessary for a simultaneously performed study. Subsequently the micropigs were bled and perfused with saline. The hearts and more distal parts of the aorta were excised. From the coronary system pieces of LCA and RCA were prepared. Coronary arteries and aorta were embedded in Tissue Tek and subsequently frozen in liquid nitrogen. From rats (*n*=3), whole hearts were excised, embedded in Tissue Tek and frozen in liquid nitrogen. From all unfixed tissues, serial cryosections of approximately 10 µm were cut and collected on slides, air dried and stored at –80°C until used.

Immunohistochemistry

Slides were equilibrated to room temperature and rehydrated in phosphate-buffered saline (PBS) for 5 min. The unfixed cryosections were incubated in 0.2% Triton X-100 in PBS for 1 h and subsequently preincubated in 2% bovine serum albumin (BSA) in PBS for 30 min. Sections were incubated overnight at room temperature with appropriate dilutions of primary antibodies in PBS including 10% normal serum from the host of the secondary antibody. For labelling of gap junction proteins, antibodies raised against synthetic peptides of Cx37, Cx40 and Cx43 were used. Anti-Cx37, directed to residues 315–331 of mouse Cx37 (Willecke et al. 1991) was raised in rabbit and purified as previously described (Gros et al. 1994). On sections, 2–4 µg/ml anti-Cx37 (Delorme et al. 1997) was used. The anti-Cx40 which is directed to residues 335–356 of rat Cx40 (Beyer et al. 1992; Haefliger et al.1992) was used at a dilution of 3–5 µg/ml (Gros et al. 1994). For the labelling of Cx43, a 1 µg/ml dilution was used of a monoclonal antibody directed to a synthetic peptide corresponding to residues 252–270 of rat Cx43 (Beyer et al. 1987). The antibody (clone: Z039) was obtained from Zymed Laboratories (San Francisco, Calif., USA). Smooth muscle cells were specifically labelled with a monoclonal antibody directed to desmin (MON 3001–clone 33, 1:50; Monosan, Uden, The Netherlands). For double labellings a mixture of monoclonal and polyclonal primary antibodies was applied.

After approximately 16 h of incubation, the slides were thoroughly rinsed 3 times for 5 min with PBS and again preincubated with 2% BSA in PBS for 30 min. Fluorescence labelling was carried out in 2.5 h, using 1:100 dilutions of either fluorescein isothiocyanate- or Texas red-conjugated secondary antibodies raised against mouse or rabbit IgGs (Jackson ImmunoResearch Laboratories, West Grove, Pa., USA), diluted in PBS including 10% serum of the host of the secondary antibody. Finally, slides were thoroughly washed in PBS and mounted with Vectashield (Vector Laboratories, Burlingame, Calif., USA). To check the specificity of the labelling, sections were either incubated with the secondary antibody only, or with preimmune serum as a substitute for the primary antibody, or with a mixture of preincubated primary antibody and immunogenic peptide (15–50 µg/ml). Immunolabelled sections were examined by epifluorescence light microscopy (Nikon Optiphot-X2) and photographs were taken using Kodak 3200 ASA/TMZ black and white film.

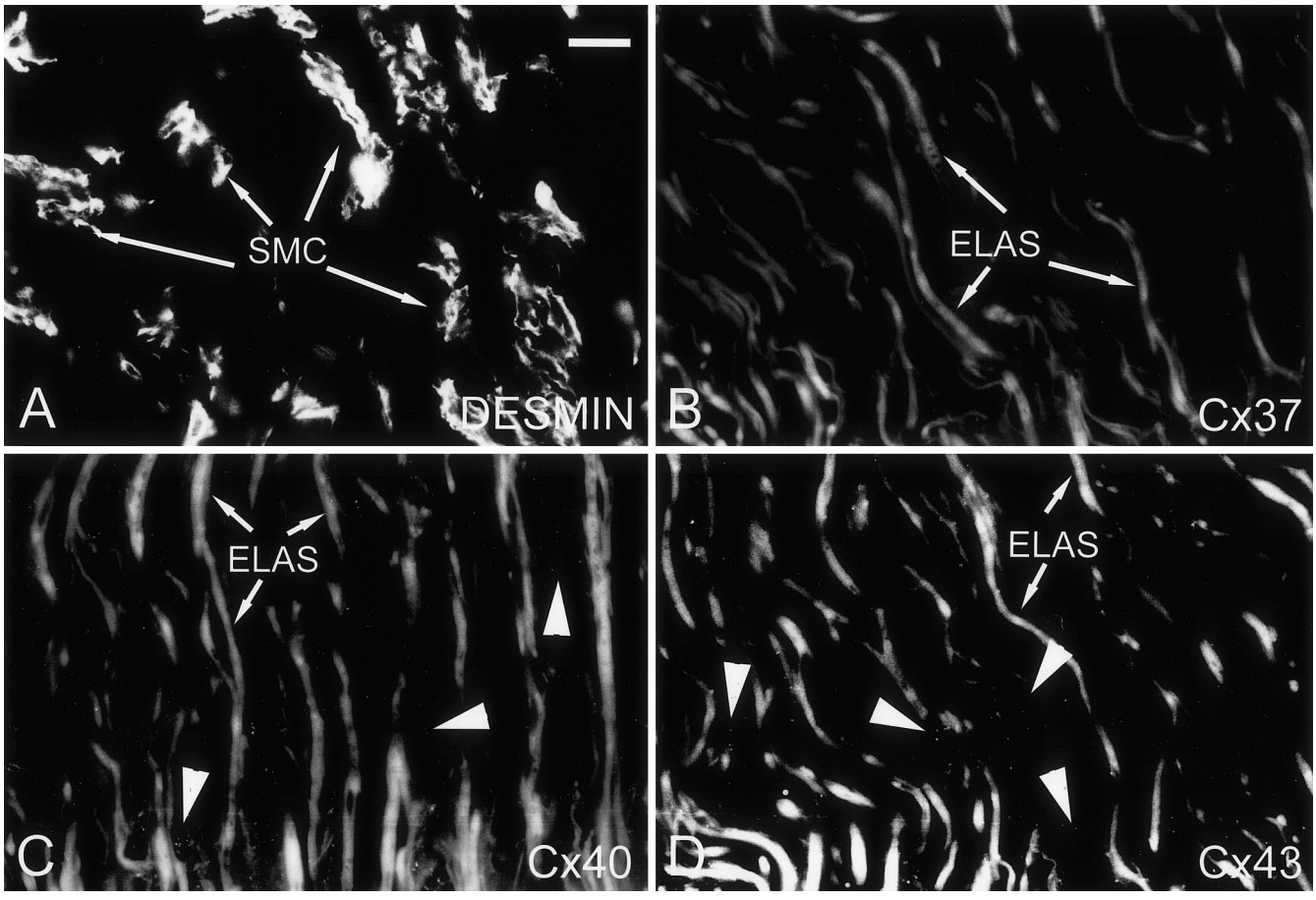

Fig. 2A–D Serial sections of bovine aorta. Gap junction labelling is represented by *white dots* and *speckles*. The photomicrograph shows a detail of the media. Elastic fibres (*ELAS*) can be distinguished as autofluorescent lines, in between which smooth muscle cells can be observed, marked by desmin (**A**). No Cx37 can be detected (**B**), while some Cx40 and Cx43 staining can be observed (*arrowheads* in **C,D**, respectively). *Bar* 25 µm

Results

Crossreaction and affinity of the antibodies with rat, micropig and cow were checked using heart tissue as positive control. In all three species, Cx37 consistently labelled between the endocardial cells in the atria, Cx40 labelled between the endothelial cells in arterioles and Cx43 labelled between both atrial and ventricular myocytes. All with apparently the same intensity.

Aorta

The elastic character of the aortic wall is well demonstrated by the abundance of elastic fibres (Figs. 1, 2, 3). In between the collagenous tissue and discontinuous sheets of elastin, which are marked by high levels of autofluorescence, relatively few smooth muscle cells can be distinguished (Fig. 2A), as can be observed from the expression of desmin. Between the smooth muscle cells of the rat aorta only labelling with the anti-Cx43 antibody can be distinguished (Fig. 1C). Due to the arrangement of smooth muscle cells and the thickness of the sections, punctated fluorescence can be recognised homogeneously over the medial layer and apparently randomly distributed in between the elastin sheets without exactly demarcating cell borders. No labelling of either the Cx37 or the Cx40 antibody can be detected between the smooth muscle cells of the rat aorta (Fig. 1A,B, respectively).

In contrast, between the smooth muscle cells of the bovine (Fig. 2) and micropig (Fig. 3) aortic medial layer, besides Cx43 (Figs. 2D, 3C), labelling of Cx40 (Figs. 2C, 3B) can be observed, also distributed in a homogeneous and apparently random pattern. As in the rat, the anti-Cx37 antibody showed no specific reaction in the smooth muscle cells of cow and micropig either (Figs. 2B, 3A, respectively).

The tunica intima of the aorta consists of a single layer of flattened endothelial cells supported by a layer of collagenous tissue rich in elastin, the elastica interna. Labelling of connexins between the aortic endothelial cells differs between the three species studied. Between the endothelial cells of rat aorta, labelling of Cx37 (Fig. 1A), Cx40 (Fig. 1B) and Cx43 (Fig. 1C) could be observed. In contrast, between bovine aortic endothelial cells, only

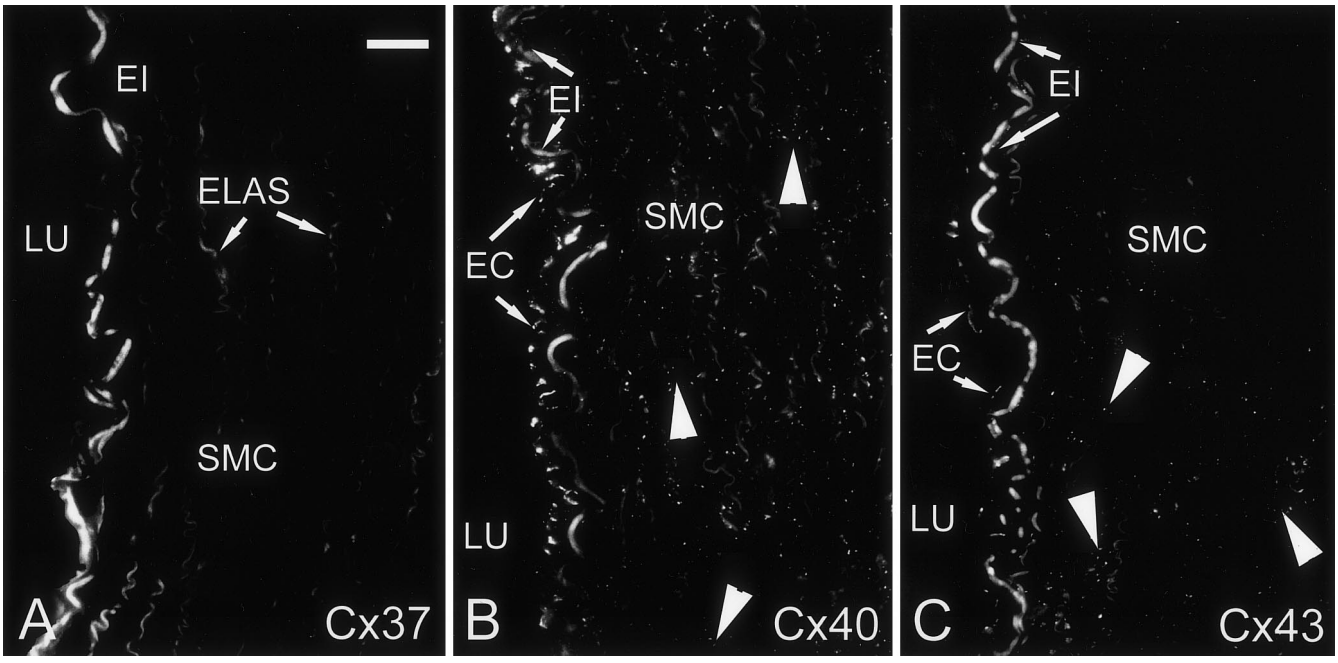

Fig. 3A–C Serial sections of porcine aorta. Gap junction labelling is represented by *white dots* and *speckles*. The photomicrograph shows part of the intima with the endothelial lining (*EC*) and the media with smooth muscle cells (*SMC*). Both layers are separated by an elastic sheet, the elastica interna (*EI*), discernible as a white autofluorescent ribbon. No Cx37 labelling can be detected between the endothelial and the smooth muscle cells (**A**), whereas Cx40 (**B**) and Cx43 (**C**) labelling can be discerned between both the endothelial (*arrows*) and the smooth muscle cells (*arrowheads*). *ELAS* Elastic fibres, *LU* lumen. *Bar* 25 µm

Table 1 Comparison of distribution patterns of Cx37, Cx40 and Cx43 in the aorta and coronary system of cow, micropig and rat

Blood vessel	Cell	Cx37	Cx40	Cx43
Cow				
Aorta	Endothelial	–	+	–
	Smooth muscle	–	+	+
Coronary artery	Endothelial	–	+	+/–
	Smooth muscle	–	+	+/–
Micropig				
Aorta	Endothelial	–	+	+
	Smooth muscle	–	+	+
Coronary artery	Endothelial	+	+	+
	Smooth muscle	–	+	+
Rat				
Aorta	Endothelial	+	+	+
	Smooth muscle	–	–	+
Coronary artery	Endothelial	+	+	–
	Smooth muscle	+	+	+

Cx40 labelling can be observed between the endothelial cells, whilst the staining for Cx43 and Cx37 is negative (not shown). In the micropig, labelling between the aortic endothelial cells can be observed with both the Cx40 (Fig. 3B) and the Cx43 (Fig. 3C) antibody, whereas labelling for anti-Cx37 is negative (Fig. 3A).

Coronary artery

Muscular arteries have the same basic structure as elastic arteries but the amount of elastic tissue is greatly reduced while the muscle component is of relatively greater importance and recognisable as a layer of circumferentially or longitudinally arranged smooth muscle cells. In the larger arteries of the rat coronary system all three connexin isotypes can be detected between the smooth muscle cells (Fig. 4). For both Cx40 and Cx43, the labelling intensity is low (Fig. 4B,C) while Cx37 is easy recognisable (Fig. 4A). Between the smooth muscle cells in the smaller arterioles, none of the connexin isotypes we were staining for could be detected. In the micropig, both Cx40 and Cx43 but not Cx37 could be detected in the media. Between the smooth muscle cells in the proximal parts of the bovine coronary arteries, we could not detect Cx37 or Cx43, while Cx40 is readily detectable. In bovine arteries that are located more distally in the coronary tree, Cx43 can be detected between smooth muscle cells at sites where vessels bifurcate (Fig. 5C,D), while the expression of Cx40 (Fig. 5A) is comparable with the level of expression that is observed between smooth muscle cells in the proximal part of the coronary system.

The intimal lining of the coronary system comprises one layer of endothelial cells which is directly bordered by the internal elastic lamina. In rat both Cx37 and Cx40 labelling can be detected between endothelial cells (Fig. 4A,B), while staining for Cx43 was negative (Fig. 4C). Between the micropig coronary endothelial cells all three connexin isotypes showed specific fluorescent staining, most abundantly for Cx40 and Cx43, while Cx37 showed a more heterogenous staining pattern (not

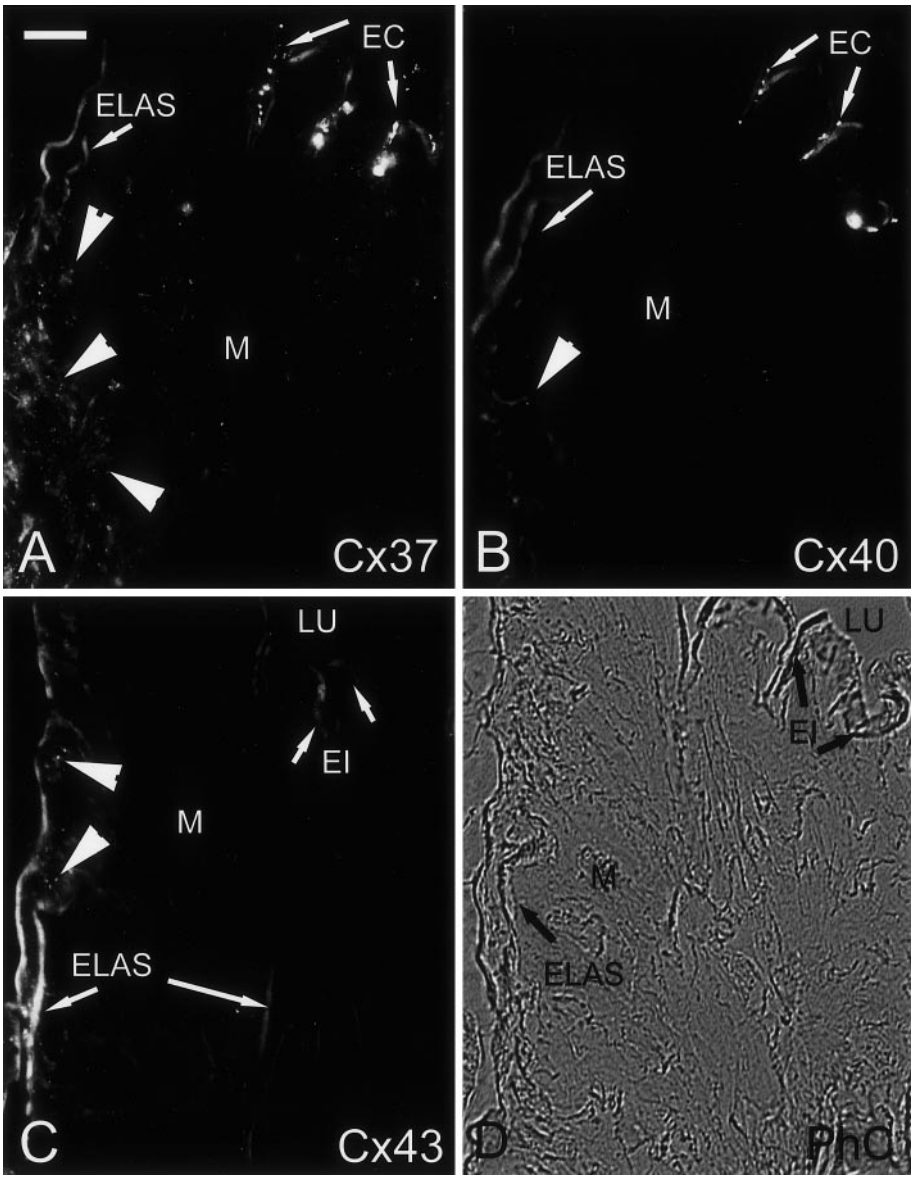

Fig. 4A–D Serial sections through rat coronary artery. Gap junction labelling is represented by *white dots* and *speckles*. **A–C** Consecutive immunolabelled sections. Cx37 (**A**) and Cx40 (**B**), but not Cx43 (**C**) labelling can be observed between the endothelial cells (*EC*; *arrows*). Between the smooth muscle cells of the media (*M*) all three connexin isotypes can be detected (**A–C** *arrowheads*), with highest staining intensity at the border of the elastica externa. Labelling intensities for both Cx40 (**B**) and Cx43 (**C**) are low, while Cx37 staining (**A**) is easy recognisable. **D** Phase contrast representation from a cross-section showing the lumen and endothelial lining in the *right-hand corner*, the media with smooth muscle cells in the middle and the elastica externa at the *left side* of the picture. *ELAS* Elastic fibres, *LU* lumen, *EI* elastica interna. *Bar* 25 µm

shown). In the bovine coronary system only Cx40 is always detectable between endothelial cells (Fig. 5A). While Cx43 is present at low levels between endothelial cells in the proximal part of the vascular tree, in the more distally located vessels, Cx43 could be detected more abundantly at sites near a bifurcation (Fig. 5C,D). Cx37 could never be observed between endothelial cells in the bovine coronary system. Table 1 shows an overview of the described expression patterns of all three connexins in the aorta and coronary artery in cow, micropig and rat.

Discussion

Reaching consensus with regard to expression of connexin isotypes in vessel wall cells is difficult due to the variation in species and vessel types investigated (Beny and Connat 1992; Beyer et al. 1992; Bruzzone et al. 1993; Gabriels and Paul 1998; Larson and Haudenschild 1990; Little et al. 1995; Polacek et al.1993; Reed et al. 1993; Rennick et al. 1993; Van Rijen et al. 1997; Yeh et al. 1998). It becomes even more complex when connexin expression patterns in situ are compared with those described in vitro. Pursuing consensus for distribution profiles of typical vessel wall connexins (Cx37, Cx40 and Cx43), we performed a comparative study on their distribution in healthy rat, micropig and bovine aorta and coronary artery, which represented conduit- and resistance-type of vessels, respectively.

Connexin37

Although crossreactivity of the anti-Cx37 antibody with rat, micropig and bovine Cx37 was confirmed by consis-

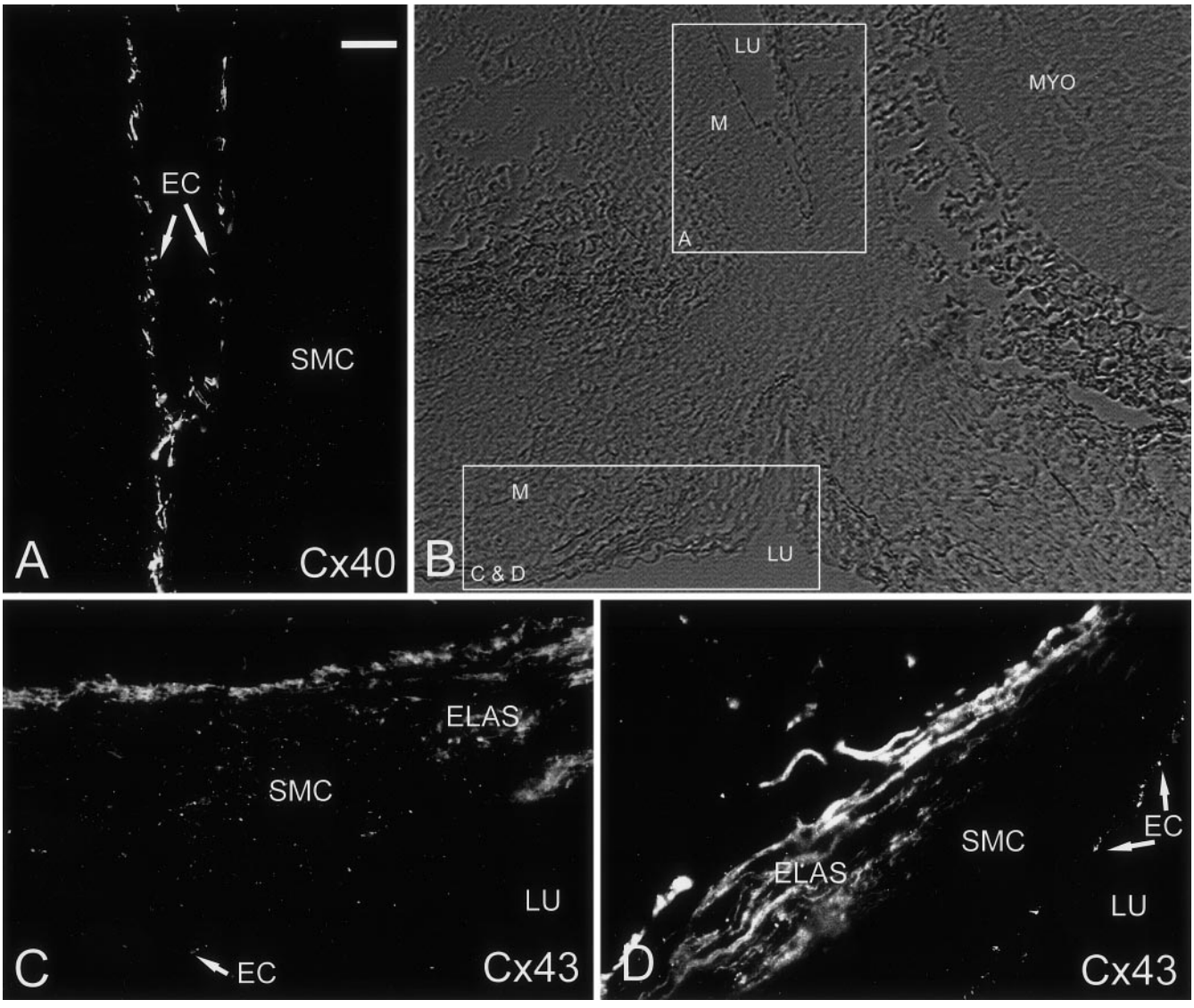

Fig. 5A–D Serial sections through distally located bovine coronary artery. **A,C,D** Consecutive immunolabelled sections. They present the respective *boxed areas* of **B** at higher magnification. **B** Phase contrast overview of a section showing the vessel wall near a bifurcation (*lower boxed area* **C,D**) to another branch in the coronary system (*upper boxed area* **A**). Gap junction labelling is exemplified by *white dots* and *speckles*. Cx40 can be readily detected between the endothelial cells (*EC*; **A** *arrows*) and the smooth muscle cells (*SMC*). Cx43 can be detected between the endothelial cells (**C,D** *arrows*) with higher labelling intensity in the proximity of the bifurcation (**D**). Cx43 can also be detected between the smooth muscle cells of the media (**C**). *ELAS* Elastic fibres, *LU* lumen, *MYO* myocardium. *Bar* 25 µm

tent positive fluorescent labelling of the atrial endocardium, the anti-Cx37 antibody only labels cells abundantly in rat vasculature, between the endothelial cells of the aorta and both the endothelial cells and smooth muscle cells of the coronary artery. The expression of Cx37 protein between endothelial cells of rodents, both in situ and in vitro may be considered commonly accepted (Delorme et al. 1997; Gabriels and Paul 1998; Ko et al. 1999a; Yeh et al. 1998). However, we could also detect Cx37 labelling between smooth muscle cells, but only in rat coronary artery and not aorta. Reed et al. (1993) used freshly isolated rat aortic smooth muscle cells and the A7r5 smooth muscle cell line from rat aorta to determine the expression pattern of Cx37 mRNA, but were also not able to detect this connexin in these cells. This suggests Cx37 expression to be species and site specific.

Less obvious labelling was observed between the endothelial cells in the coronary artery of the micropig which is consistent with previous findings of Cx37 mRNA expression in freshly isolated and cultured porcine aortic endothelial cells (Carter et al. 1996). Although the expression of Cx37 mRNA in primary cultures of bovine endothelial cells has also been reported (Reed et al. 1993), we were not able to detect Cx37 protein in bovine vasculature. Considering the faint labelling between pig endothelial cells it may well be that Cx37 protein in cow is expressed in quantities below the detection level of immunohistochemistry.

Connexin40

We are able to detect anti-Cx40 antibody labelling consistently between both endothelial and smooth muscle cells in rat, micropig and bovine vasculature. The only exception is formed by smooth muscle cells in rat aorta, where no labelling was observed. Although it can be concluded from this study that Cx40 is usually present between the smooth muscle cells of the media in situ, Ko et al. (1999b) could not detect any immunolabelling of Cx40 protein in the media of the elastomuscular human mammary artery. Most studies that report on the distribution of Cx40 in the vessel wall in situ, describe exclusively the expression of this connexin between endothelial cells (Bruzzone et al. 1993; Gros et al. 1994; Ko et al. 1999a; Yeh et al. 1998), however Cx40 protein is reported to be expressed between smooth muscle cells as well (Little et al. 1995; Van Rijen et al. 1997). The neglect of Cx40 immunostaining between smooth muscle cells may be partly explained by the differences in morphology between endothelial and smooth muscle cells and the subsequent disparities in cellular localisation of connexin staining between both cells. Moreover, the contribution of Cx40 to endothelial cells is prominent (Ko et al. 1999a) and hence immunolabelling is easily identifiable as dense lines between the large cross-sectioned cells, however gap junctions between smooth muscle cells are smaller and more dispersed over the membrane of the spindle-shaped cells.

In vitro, Cx40 protein or mRNA could only be detected temporally between vascular smooth muscle cells (Beyer et al. 1992; Reed et al. 1993) and endothelial cells (Van Rijen et al. 1997). After several culture passages, Cx40 expression gradually disappeared probably due to increasing deficiencies of culture components.

Connexin43

Only in the pig could Cx43 be commonly detected between endothelial and smooth muscle cells of the aorta and coronary artery. In agreement with observations of Yeh et al. (1997, 1998), in the rat, we observed Cx43 antibody labelling between the endothelial cells of the conduit vessel but not between the endothelial cells of the resistance vessels. Aortic smooth muscle cells were highly positive for Cx43 staining and also some staining could be observed between smooth muscle cells of the coronary artery. Between bovine aortic smooth muscle cells Cx43 labelling could be consistently detected. We were not able to locate specific labelling between the bovine aortic endothelial cells, which is in contrast with Reed and Beyer (Beyer et al. 1992; Reed et al. 1993) who did observe Cx43 mRNA in (freshly) isolated endothelial cells. However, in the bovine coronary vasculature, not only between smooth muscle cells but also between endothelial cells, Cx43 could be detected. Near ramifications in the coronary tree, Cx43 labelling is more abundant as compared to the rest of the vessel, which coincides with the observation of Gabriels who show that Cx43 protein between endothelial cells in the rat aorta is highly localised to sites of disturbed flow such as ostia of branching vessels and at flow dividers (Gabriels and Paul 1998). Furthermore, Cowan et al. (1998) observed an upregulation of Cx43 mRNA expression in the vessel wall by mechanical loads.

We can only speculate what the functional implications of the diversity and differential distribution of connexins in the vasculature may be. The specific localisation of connexins each with individual connexin isotype-specific properties may serve to compartmentalise groups of cells and restrict cell–cell communication. Although much of the individual biophysical and biochemical properties of connexins are characterised, to date it is unknown what the specific function of an individual connexin in a given environment is. Cx37-, Cx40- and Cx43-knockout mice have been developed and are currently under study (Kirchhoff et al. 1998; Reaume et al. 1995; Simon et al. 1997, 1998). As for the Cx37- and Cx43-knockout mice, no phenotype concerning the vasculature has been reported so far. Recently, in the Cx40-knockout mouse, an increase in blood pressure was measured suggesting a regulatory role for Cx40 in the coordination of vasodilation (C. De Wit unpublished observations).

Recently, some data were presented concerning a double Cx37/Cx40-knockout mouse (A.M. Simon unpublished observations). Double homozygotes die perinatally, and almost all animals have visible surface bruises on the dorsal head, along the back or on the posterior region of the animal. Histological sections revealed the presence of extravascular blood in these areas. These results suggest a breakdown in vascular integrity in Cx37/Cx40 double knockout mice of which the nature and extent is at present unclear.

From this study we conclude that Cx40 is the most commonly expressed gap junction protein between endothelial cells and smooth muscle cells in arteries, except from rat aorta smooth muscle cells. Cx43 is usually expressed between smooth muscle cells, and between endothelial cells expression is differentiated among vessels and species. Since Cx40 and Cx43 fail to establish functional channels (Bruzzone et al. 1993; Haubrich et al. 1996), their spatially differentiated distribution patterns may represent functionally separated physiological processes. Cx37, when expressed, is mostly confined to endothelial cells, although we observed some labelling between smooth muscle cells of rat coronary artery. It would allow coupling of endothelial specific processes and/or form heterotypic gap junctions with Cx40 and Cx43 to establish cell communication and regulate processes which do not require compartmentalisation.

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